**Immunopathology and Infectious Diseases**

Gingival Lymphatic Drainage Protects Against *Porphyromonas gingivalis*–Induced Bone Loss in Mice

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Periodontitis is characterized by tissue destruction and bone loss mainly due to inflammatory responses after bacterial challenge of the gingiva. Gingiva is supplied with lymphatics that drain interstitial fluid and transport immune cells to the lymph nodes for antigen presentation; yet, the role of lymphatics in periodontal disease development is unknown. To investigate the lymphatic function after periodontal infection, we used K14-VEGF receptor 3-Ig (K14) mice that lack lymphatics in gingiva. Mice were orally infected with human *Porphyromonas gingivalis* and observed for 42 days. The infected K14 mice developed significantly more bone loss than the wild-type mice, and were associated with an increased number of macrophages and major histocompatibility complex class II antigen-presenting cells in the bone resorptional areas. The infected transgenic mice expressed a significant higher periodontal level of several proinflammatory cytokines, whereas the plasma level of *P. gingivalis* IgG was significantly lower than in the wild-type mice. No differences were found in immune cell distribution in draining lymph nodes between the strains. Our results show that a strong periodontal inflammatory response and a weakened systemic humoral B-cell response took place in K14 mice after infection. We conclude that gingival lymphatics protect against *P. gingivalis*–induced periodontitis, and we speculate that they are critical in the protection by clearance of infection and by promotion of humoral immune responses. (Am J Pathol 2012, 181:907–916; http://dx.doi.org/10.1016/j.ajpath.2012.05.027)

Infectious periodontal disease is accompanied by chronic inflammation of the gingiva and may cause destruction of periodontal tissue followed by alveolar bone loss. The disease is the most prevalent form of bone pathology in humans, with a frequency of 10% to 15% in the adult population.

The periodontium is chronically exposed to large numbers of bacteria. *Porphyromonas gingivalis* (*P. gingivalis*), a black-pigmented Gram-negative anaerobic bacterium, is one of the major periodontopathic bacteria strongly associated with the disease. These periodontopathic bacteria produce toxic products that induce host responses, including production of proinflammatory cytokines and recruitment of inflammatory cells. This response is meant to eliminate the microbial challenge rather than the bacterial toxic products that are thought to be the immediate cause for periodontal breakdown. The immune responses set up in gingiva include both innate and adaptive responses. Dendritic cells in oral mucosa detect foreign antigens and migrate into regional lymph nodes, where they stimulate antigen-specific T- and B-cell proliferation, thereby initiating adaptive immune responses. The lymphocytes migrate to the tissue, attracted by locally produced chemokines and cytokines; and in the peripheral tissue, they induce targeted immune responses.

In addition to immune cell surveillance, the lymphatic vasculature is important for maintenance of tissue fluid homeostasis by collecting protein-rich fluid from the extracellular spaces and for clearance of foreign invaders and their bacterial products. Initial lymphatics originate in the interstitium, are blind ended, and are made of a single-layer lymphatic endothelial cell sheet with loose junctions that absorb lymph fluid and cellular infiltrate. In the gingiva, initial lymphatic vessels are found in the connective tissue under the epithelium and also in the junctional epithelium. Their endothelium expresses CCL21, a chemoattractant for lymphocytes and mature dendritic cells, and CCL21 induction occurs when Toll-like receptor 4 expressed on initial lymphatics recognizes and is engaged by lipopolysaccharide of Gram-negative bacteria.

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Previously, the peripheral lymphatic system was looked on as a passive transport system for fluid removal and immune cell transport, but more recent evidence shows that the peripheral lymphatic system plays an active role in development of chronic inflammatory diseases.\textsuperscript{13,14} We have recently shown that lymphatic vessels grow during periodontal disease development,\textsuperscript{15} but the role of lymphatic vessels in the pathogenesis of such disease has so far never been investigated.

Animal models, and mouse models in particular, are useful in studying the capacity of the host immune response to protect or contribute to periodontal disease.\textsuperscript{16,17} We used K14-VEGFR-3-Ig (K14) mice\textsuperscript{18} that completely lack lymphatic vessels in gingiva and are practically depleted of lymphatics in the mucosal part of the alveolar mucosa.\textsuperscript{19} Accordingly, we infected K14 mice with \textit{P. gingivalis} to observe whether the lack of lymphatic vessels in gingiva may cause altered immune responses and influence the bone loss. We hypothesized that lack of gingival lymphatics would reduce the capacity to remove bacteria and their products from the gingiva and thereby induce a more severe infection in gingiva followed by more marginal bone resorption.

In agreement with our hypothesis, we here show that transgenic mice (K14) lacking gingival lymphatics have more periodontal bone resorptive activity, stronger inflammatory immune responses, and also weakened humoral immune responses after \textit{P. gingivalis} challenge than their wild-type (WT) littermates. Our observations suggest that gingival lymphatic drainage protects against development of periodontal disease characterized by marginal bone loss.

\section*{Materials and Methods}

\subsection*{Animals}

Experiments were performed in 13- to 17-week-old K14-VEGFR-3-Ig (K14) mice of C57/B16 background and, as controls, C57/B16 wild-type mice. The K14 mouse model has a complete lack of gingival lymphatics,\textsuperscript{19} and was generously provided by Dr. Kari Alitalo (Molecular/Cancer Biology Laboratory and Haartman Institute, University of Helsinki, Helsinki, Finland). Mice were genotyped by polymerase chain reaction as previously described.\textsuperscript{19} They were fed a standard pellet diet and tap water \textit{ad libitum} before experiments. All procedures were performed in accordance with the guidelines of the Norwegian State Commission for Laboratory Animals and were approved by the local ethical committee.

\subsection*{Bacteria}

\textit{P. gingivalis} ATCC 53978 (W50; American Type Culture Collection, Manassas, VA) was cultivated on fastidious anaerobe agar (Lab M, Bury, UK), supplemented with 5% sterile defibrinated sheep blood, and incubated anaerobically (10% CO$_2$, 5% H$_2$, 85% N$_2$) at 37°C for 5 days.

\section*{Oral Infection}

In both strains of mice, one group \((n = 7)\) was infected (experimental) and one group \((n = 7)\) was sham treated (controls). Periodontal infection was induced as described previously.\textsuperscript{16} Briefly, animals were given sulfamethoxazoletrimethoprim, 10 mL per pint in deionized water, \textit{ad libitum} for 10 days to reduce the original oral flora, followed by a 4-day antibiotic-free period. Experimental animals were given 10$^9$ colony-forming units of \textit{P. gingivalis} suspended in 100 $\mu$L of PBS with 2% carboxymethylcellulose (Sigma-Aldrich, St. Louis, MO) placed into the esophagus and oral cavity three times at 2-day intervals. Controls included sham-infected mice that received the antibiotic pretreatment and the carboxymethylcellulose gavage without \textit{P. gingivalis}. Forty-two days after the last gavage, blood samples were collected by cardiac puncture from each mouse and the serum separated and stored at –80°C until analyses for serum IgG. This duration of the experiment was used because it has been sufficient to demonstrate periodontitis in other mouse models.\textsuperscript{20,21} All jaws (maxilla and mandible) were collected and handled as described below.

\subsection*{Serum Antibody to \textit{P. gingivalis}}

The level of serum antibody specific to \textit{P. gingivalis} ATCC 53978 (W50) was measured as described previously.\textsuperscript{16} Briefly, 96-well polystyrene plates (Nunc, Roskilde, Denmark) were coated with formalin-killed \textit{P. gingivalis} ATCC 53978 (W50) overnight at 4°C. Serial dilutions (2$_p$ to 2$_{12}$) of test or positive control sera were incubated overnight at 4°C and washed. Alkaline phosphatase–conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) was used to detect bound antibody, which was quantified by p-nitrophenyl phosphate substrate (Sigma-Aldrich). Absorbance was determined at an optical density at 450 nm by microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA). Titer was defined as the highest dilution factor that gave the optical density reading more than 0.1 OD units above background.

\subsection*{Alveolar Bone Loss}

Skulls were boiled 10 minutes in distilled water, defleshed, immersed overnight in 3% hydrogen peroxide, pulsed for 1 minute in bleach, and stained with 1% methylene blue. Images from one side with maxillary molar teeth and alveolar bone were captured using a dissection microscope (Model C-DSD 230; Nikon Instruments, Tokyo, Japan) equipped with a digital camera (WD 70; Nikon Instruments) and saved as JPEG files. An image of a precise ruler was captured at the same magnification and used for the calibration. The area of periodontal bone loss was determined using the polygonal area enclosed by the cementoenamel junction, the lateral margins of the exposed first and third molars’ roots, and the alveolar ridge. The areas circumscribe the exposed root surfaces. Areas were measured using Lucia imaging software (Lucia v. 480; Laboratory Imaging, Hostiva, Czech Republic). Measurements were done by two inde-
Osteoclast Staining

Osteoclasts were identified using an Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich). Osteoclast number was counted in a grid (100 × 200 μm) placed on top of the approximal bone between the molars in three to five sections from each animal, and reported as TRAP+ cells per grid area.

Immunohistochemical Immune Cells Staining

For evaluation of immune cells, lower jaws were decalcified in EDTA for 4 days, immersed in 30% sucrose overnight, and stored at −80°C. Jaws were sectioned (8 to 9 μm) and fixed in cold aceton for 10 minutes before immunostaining was done. A standard three-stage avidin–biotin peroxidase method was used for detection of immune cells. Briefly, quenching of endogenous peroxidase activity was performed by exposing the sections to 0.3% H2O2 in 90% methanol for 30 minutes, followed by incubation with rabbit serum for 3 hours to block nonspecific tissue binding. The following primary antibodies were used: goat anti-mouse CD20 (dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-mouse Ly-6B.2 (dilution 1:4000; AbD Serotec, Kidlington, UK), rat anti-mouse CD4 (dilution 1:100; AbD Serotec), rat anti-mouse CD8 (dilution 1:50; AbD Serotec), rat anti-mouse F4/80 (dilution 1:1000; Acris Antibodies, Hiddenhausen, Germany), or rat anti-mouse major histocompatibility complex (MHC) class II (dilution 1:1500; eBiosciences, San Diego, CA). Antigen–antibody complexes were detected by the avidin–biotin peroxidase (ABC) method, using a commercially available kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA), and visualization of the antigen–antibody reaction was done using 3,3′-diaminobenzidine (Sigma-Aldrich) in the presence of nickel. The sections were counterstained with methylene blue/azure II in 1% sodium borate and distilled water, dehydrated in a graded alcohol series, cleared in xylene, and coverslipped using Eukitt (O. Kindler, Freiburg, Germany). Isotype-matched IgG from their respective companies served as negative controls.

Immune Cell Counting

Counting was performed using a ×40 magnification in a 100 μm × 150 μm grid under the Nikon photomicroscope (Nikon Eclipse E600; Nikon Instruments) connected to a digital camera using Lucia imaging software (Lucia v. 480; Laboratory Imaging). Positively stained cells were counted in lamina propria adjacent to the alveolar bone (mesially and distal to second molar, and in the mesial papilla to the first molar) in three to five sections from each animal. The total number of positive-stained cells in the grid area was counted and reported as cells per area.

Analysis of Cytokines

For protein extraction, periodontal tissues (buccal and palatal tissue from one maxillary quadrant) were collected and snap frozen in liquid N2, and stored at −80°C until analyzed. The samples were homogenized in 600 μL of lysis buffer added to 1 μL/mL aprotinin and leupeptin (both from Sigma-Aldrich) together with 50 μg/mL gentamicin (Garamycin; Essex Pharmaceuticals, New York, NY) in a Mixer Mill 301 (Retsch, Haan, Germany). The samples were centrifuged at 15,300 × g at 4°C, and the supernatants were stored at −80°C. The concentrations of cytokines in periodontal tissue were determined using a multiplex kit (Millipore, Billerica, MA).

A panel of the following cytokines was simultaneously quantified with multiplex analysis: granulocyte-colony stimulating factor (G-CSF), interferon-γ (IFN-γ), interleukin (IL)-10, IL-17, IL-1α, IL-2, IL-6, inducible protein (IP)-10, keratinocyte-derived chemokine (KC), macrophage-colony stimulating factor (M-CSF), macrophage inflammatory protein (MIP)-1α, regulated on activation normal T cell expressed and secreted (RANTES), and tumor necrosis factor (TNF)-α. Total surface fluorescence was measured with a flow-based dual laser system (Luminex 100; Luminex, Austin, TX) for the detection of different color-coded beads and quantitation of cytokines. Cytokine concentration was calculated with reference to a standard curve based on a broad range of standards (4.8 to 20,000 pg · mL−1) provided in the kit. To control and verify the multiplex results, enzyme-linked immunosorbent assay measurements were performed (R&D DuoSets; R&D Systems, Minneapolis, MN) in accordance with the manufacturer’s instructions.

Flow Cytometry of Immune Cells from Cervical Lymph Nodes

Cervical lymph nodes were dissected free and incubated with 0.28 Wünsch units/mL of Liberase Thermolysin medium (Roche-F. Hoffmann-La Roche, Basel, Switzerland) for 50 minutes at 37°C, to liberate leukocytes according to the manufacturer’s instructions. For immunofluorescence staining, the cells were filtered through a 70-μm filter and then washed three times in PBS and incubated for 30 minutes at 4°C with the optimal dilution of each antibody. The following conjugated antibodies were used for antigen detection: Pacific Blue CD4 (1:25), PE CD8 (1:25), APC CD19 (1:50), PerCP-CY5.5 CD11c (1:25) (all BD Biosciences, San Jose, CA), FITC F4/80 (1:25) (Acris Antibodies), and Alexa Fluor 700 Ly-6B.2 (1: 10) (AbD Serotec). Cells were washed again, and approximately 300,000 cells were analyzed by flow cytometry (FACScan and CELLQuest software; BD Biosciences). Negative controls were unstained cells from lymph nodes; and for control of gate settings, each antibody was incubated with microparticles from an anti-rat Ig, κ/negative Control Compensation Particles Set (BD Biosciences). Results represent the percentage of isolated cells in the cervical lymph nodes.
Data Analysis

Results are given as means ± SE. Data were analyzed with one-way analysis of variance followed by Holm-Sidak or Bonferroni post hoc tests. *P < 0.05 was considered statistically significant.

Results

Increased Alveolar Bone Loss in K14 Mice

To investigate whether the lack of lymphatics affected bone loss, we inoculated mice orally with live P. gingivalis, and alveolar bone loss was determined on day 42 after infection. As shown in Figure 1, infected K14 mice exhibited more alveolar bone loss than the infected WT littermates, as the exposed area was significantly increased in the former compared with the latter (Figure 1, A and B). The infected K14 mice showed more bone loss than their own sham control mice, in contrast to the WT mice in which the infection did not stimulate bone loss (Figure 1, A and B). These data show that significant bone mass reduction was induced after P. gingivalis infection in K14 mice only, suggesting that the lack of lymphatics results in increased bone loss.

Because of the difference in bone degradation, we next used TRAP staining to determine whether the number of osteoclasts differed between the strains. Histological analysis (Figure 2A) showed no statistical difference in the number of TRAP⁺ cells on the alveolar bone surface of K14 infected mice compared with WT infected (P = 0.07) as well as K14 shams (Figure 2B). The alveolar bone surface in K14 infected mice was irregular, with bone resorption lacunae (Figure 2A).

Weaker Humoral Immune Responses in K14 Mice

We next studied the humoral response to infection in the two strains. Serum P. gingivalis IgG levels were significantly elevated in both strains of mice challenged with P. gingivalis compared with their respective shams (P <
0.001) (Figure 3). Interestingly, the IgG titers in K14 infected mice were significantly lower (\( P < 0.05 \)) compared with WT infected mice, demonstrating that the humoral immune response that developed in K14 was weaker than in WT mice. No differences were observed between the sham-treated mice (Figure 3).

### Increased Immune Cell Infiltration in Gingiva after P. gingivalis Infection in K14 Mice

To study immune cell infiltration, we stained for various immune cells. In the K14 infected mice, strong infiltration of F4/80 cells were found in bone resorption lacunae as illustrated in Figure 4A. Moreover, the number of F4/80 cells in lamina propria above the alveolar bone of infected K14 mice was significantly higher compared with infected WT mice (\( P < 0.05 \)) (Figure 4B). The number of antigen-presenting MHC class II cells adjacent to the alveolar bone were also significantly higher in K14 than in the WT mice after infection (Figure 4C), and no strain differences were found between the sham mice.

A low number of neutrophils were found toward alveolar bone after infection in both strains of mice (data not shown). However, in two infected K14 mice, a periodontal abscess was found, and neutrophils surrounded the abscess along with other infiltrating immune cells (Figure 5), suggesting a more severe infection in the K14 strain.

In the inflammatory infiltrate toward bone, numerous CD20 B cells were found in the infected K14 mouse (Figure 6A), but the total number was not significantly higher than in the infected WT mouse (Figure 6B), probably because the WT had more B cells evenly distributed in the grid area (Figure 6A). Due to technical reasons, we were not able to quantify T cell (i.e., identify CD4/CD8 immune cells) staining in the sections.

### Differential Inflammatory Mediator Responses in Periodontal Tissue after P. gingivalis Infection

To investigate whether inflammatory immune responses were different between the two strains of mice, we next quantified the cytokine/chemokine expression in the periodontal tissue after P. gingivalis infection using multiplex analysis and verified the results with enzyme-linked immunosorbent assay mea-
surements. As shown in Figure 7, significantly higher levels of IFN-γ (P < 0.05), IL-1β (P < 0.05), and G-CSF (P < 0.001) were found in K14 infected mice compared to WT infected mice. Furthermore, the concentration of the interferon gamma–IP-10 in K14 infected mice tended to be higher than in WT infected mice (Figure 7). No strain differences were found in the levels of TNF-α, IL-6, IL-10, and MIP-α (data not shown). Interestingly, M-CSF expression was significantly up-regulated in K14 sham mice compared with WT sham mice (P < 0.01), and was also high in both infected strains (Figure 7). The levels of IL-17, IL-2, KC, and RANTES were below detectable levels. Collectively, the cytokine analysis showed a stronger inflammatory response in K14 than in WT mice after infection.

No Difference in Immune Cell Distribution in Local Draining Lymph Nodes

To study whether the presence of lymph vessels in the tissue affected the draining lymph nodes, we compared the immune cell distribution in the two strains. There were fewer cervical lymph nodes in the K14 mice, and their size varied more than in the corresponding WT littermates. However, flow cytometry revealed no significant differences in immune cell distribution between the strains in any condition (Figure 8). The cell viability was higher than 80% for all lymph nodes.

Discussion

To our knowledge, these are the first data on the function of lymphatic vessels in an infectious disease that results in bone resorption. We demonstrate that the transgenic mouse model (K14-VEGF receptor 3-Ig) that lacks gingival lymphatics develops a more severe local inflammatory response in gingiva and loses significantly more alveolar bone on bacterial challenge than their WT littermates. In the inflammatory infiltrate adjacent to the alveolar bone, the number of macrophages and MHC II antigen-presenting cells were higher in the transgenic mice compared to the WT mice after infection. By contrast, the humoral immune response (serum-specific P. gingivalis IgG) was weaker in the transgenic mice.

We used a well-established model for induction of periodontitis in mice by oral inoculation of a human black-pigmented anaerobic bacterium, P. gingivalis. The C57BL/6 WT mice have previously been reported to be relatively resistant to periodontal disease induction and develop insignificant or modest bone resorption after P. gingivalis exposure. However, the transgenic mice that lack initial lymphatics were susceptible to develop periodontitis and showed significant bone loss 42 days after infection in contrast to their WT littermates.
The numbers of infiltrating immune cells (macrophages, B cells, MHC II cells) were increased in gingiva adjacent to the alveolar bone in the K14 strain after infection, although not significantly for the B cells. Macrophages are important links between the innate and adaptive immune system, and it has been demonstrated that a considerable proportion of macrophages in bony lesions may express MHC class II molecules, suggesting that they may contribute to lesion development through activation of T-cell–mediated immune responses. Macrophages can also serve as osteoclast precursors, thereby further reinforcing their potential role in periodontal disease development.

In areas with immune cell infiltration toward the bone, neutrophils were found between mononuclear cells 42 days after infection. This was particularly pronounced in infected K14 mice, and to a lesser degree in infected WT mice. Abscess formation in gingiva above the alveolar bone was also observed in infected K14 mice only, and was surrounded by infiltrating neutrophils and mononuclear cells. The accumulated neutrophils close to the alveolar bone may degranulate and release superoxide anion resulting in degradation of soft tissue and bone in line with what has been described in the pathogenesis of inflammatory arthritis. B cells in periodontal lesions are also linked to periodontal disease development together with T cells, as they contribute substantially to production of receptor activator of nuclear factor-κB ligand (RANKL), a cytokine known to induce osteoclast activity (see below). Furthermore, the K14 infected mice had significantly higher levels of the proinflammatory cytokines IFN-γ and IL-1β. IL-1β has been strongly associated with alveolar bone resorption via stimulation of RANKL expressed by osteoblasts, lymphocytes, and activated macrophages. IL-1 also promotes up-regulation of M-CSF, which stimulates the formation of osteoclast-like cells and together with RANKL cause mature osteoclast activation.

IFN-γ is anti-osteoclastogenic in vitro, but also induces inflammatory responses in vivo that overcome the inhibitory effect with eventual osteoclastogenic outcome. IFN-γ is a key Th1 cytokine and activates macrophages,
reduces macrophage-suppressive activity, and induces IL-1 production, NO synthesis, and \( \text{O}_2^- \) \text{2}–\text{3}6 During inflammation, IFN-\( \gamma \) can also be produced by other immune cells such as macrophages,\textsuperscript{37} B cells,\textsuperscript{38} and neutrophils.\textsuperscript{39}

We found that infected K14 mice had significantly increased levels of G-CSF in the gingival tissue. G-CSF plays a major role in regulating granulocyte production and prolongs neutrophil survival,\textsuperscript{40}41 increases monocyte/macrophage numbers, as well as enhances neutrophil and macrophage phagocytosis.\textsuperscript{42}43 G-CSF is produced by many cells, including fibroblasts and macrophages under the influence of inflammatory stimuli.\textsuperscript{44}

Our results in the K14 strain of mice are consistent with those of Stashenko et al\textsuperscript{45} that induced strong and polarized Th1 responses to \( P. \) \textit{gingivalis} in mice, and investigated infection-induced alveolar bone loss. They found significantly more bone loss in the Th-1–biased mice and observed that the bony lesions had high expression of IFN-\( \gamma \) and IL-1. Unfortunately, we were not able to quantify T cells in our jaw sections due to inconsistency in immune labeling, but it is likely that we had increased T cell infiltration along with B cells in the bone resorptonal areas in K14 mice after infection that contributes to the observed enhanced IFN-\( \gamma \) response. Furthermore, in the infected K14 mice, an insignificant, but elevated, level of the chemokine IP-10 was found in the periodontal tissue compared to that in infected WT mice. IP-10 protein is secreted from a variety of cells (endothelial, monocytes, fibroblasts, and keratinocytes) in response to IFN-\( \gamma \).\textsuperscript{46} In bone erosive experimental arthritis, a disease with many similarities to periodontitis, IP-10, together with RANKL, has been shown to be responsible for bone destruction and to increase the infiltration of macrophages (and CD4 T cells) into inflamed joints.\textsuperscript{47} On the basis of the findings discussed above, we may conclude that there is a stronger inflammatory response in the periodontal tissue in the K14 mice after infection than in the WT mice concomitant with increased bone resorption. Moreover, the specific serum IgG response was weaker in the mutant mice. The question is whether these findings have a common denominator.

The K14 mice lack lymphatic vessels in gingiva. However, they are present in the submucosal parts of the alveolar mucosa, although they are almost completely absent in the mucosa.\textsuperscript{19} A possible explanation might be that impaired drainage of bacteria and bacterial products from gingiva and mucosa promote a more severe inflammatory response in the K14 mice, a response that becomes more severe toward the bone as the disease progresses. This hypothesis is supported by the finding of periodontal abscesses in the K14 mice only, which indicates strongly an impaired ability to clear the ongoing infection. Our previous observation that lack of lymphatics in gingiva of K14 mice is associated with an increased interstitial fluid pressure in the normal condition as well as after \( P. \) \textit{gingivalis} lipopolysaccharide challenge, probably caused by fluid accumulation in the tissue,\textsuperscript{19} further supports this assumption. In the WT littermate, the gingival lymphatic vessel area is expanded 42 days after \( P. \) \textit{gingivalis} infection,\textsuperscript{15} and it is likely that the lymphatic flow is enhanced in this situation due to the lymphangiogenic response. If so, this will further increase the difference in bacterial clearance ability from gingiva between the two strains of mice.

The humoral immune response measured as specific IgG serum responses to \( P. \) \textit{gingivalis} in K14 mice were attenuated compared to WT mice, and it reflects the level of adaptive B-cell response set up by the host. The lower IgG in the K14 strain is probably caused by impaired transport of antigen-presenting cells from the oral mucosa toward the lymph nodes after oral inoculation and exposure of mucosa to the pathogenic bacteria. A high specific serum IgG is shown to be protective in \( P. \) \textit{gingivalis}–induced bone loss in mice,\textsuperscript{48} and might have contributed in the protection against periodontal disease development seen in the WT mice in this study.

Surprisingly, there were no statistical differences in immune cell distribution in local draining lymph nodes either within the strains or between the strains in any of the experimental situations. Despite anatomical differences in number and size of lymph nodes in K14 mice compared with WT mice, the immune cell distribution in the lymph nodes was not different between the sham mice. This observation demonstrates that existence of lymphatics in gingiva and in oral mucosa does not affect the distribution of immune cells in the local draining lymph nodes in the steady-state condition. It is likely that a reduced antigen-presenting ability in the K14 mice might have prevented or reduced the infection-induced responses in the cervical lymph nodes. In the WT mice, with intact gingival and mucosal lymphatics, the antigen presentation should be efficient after \( P. \) \textit{gingivalis} challenge, but it is possible that at 42 days after infection, the observation period was too long to observe significant immune cell responses in the draining lymph nodes.

Lymphatic function in patients with periodontitis has never been investigated, but it is known that these patients undergo interstitial matrix degradation due to production of matrix metalloproteinases and collagenases.\textsuperscript{49}50 It is likely that degradation of the connective tissue leads to gradually impaired lymphatic function as lymphatic vessels are attached to the interstitial tissue through anchoring filaments, and opens up when interstitial fluid pressure is increased.\textsuperscript{13} On the basis of the finding in the current study, we therefore speculate that impaired lymphatic drainage capacity in periodontitis patients may aggravate the disease process by reducing the ability to clear foreign invaders and by reducing the antigen presentation in lymphoid tissue.

It is still unknown whether some patients are predisposed to periodontal disease due to a defective lymphatic system.

We conclude that lymphatic vessels are protective in \( P. \) \textit{gingivalis}–induced periodontitis in mice, and we suggest that the gingival lymphatic vessels are critical to the protection by clearing bacterial products and by promoting specific humoral IgG responses.
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